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PRINCIPAL INVESTIGATOR: Geoffrey S. Ling, M.D., Ph.D.

CONTRACTING ORGANIZATION: Uniformed Services University of the Health Sciences
Bethesda, Maryland 20814-4799

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Neurotrauma constitutes 50% of combat related casualties. Among survivors, secondary injury contributes more to functional compromise than the primary traumatic event. A leading component of secondary injury is inflammation. Antiinflammatory therapy has not been exploited for central nervous system (CNS) injury because neuroinflammation is poorly understood. In this work, we provide the first *in vivo* demonstration of neuroinflammatory control. Our work identifies and characterizes interleukin-1*B*'s (IL-1) role in neuroinflammation. We demonstrate that IL-1 leads to a specific lymphocytic CSF leukocytosis. This is dose-dependent, prolonged and confined to the CNS. Pharmacokinetic analysis reveals a terminal half-life of 2 hours. This is too short to fully account for the prolonged effect. Our studies demonstrate that IL-1 leads to tumor necrosis factor *a* (TNF) release. TNF manifests later and persists. We conclude that TNF contributes to IL-1's neuroinflammatory response. IL-6 was not significantly elevated. The work thus far domesticates that the CNS is immunoresponsive and immunocompetent. It can respond in a very specific manner to IL-1 and, in response to this cytokine, release TNF. Our future work will focus on the roles of TNF and IL-6, particularly as they relate to neutrophils.

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INTRODUCTION

Neurotrauma is the leading combat injury. During the Viet Nam conflict, 15% of battle related injuries were to the CNS. The vast majority of these patients died. Among all wounded, of those who died *after reaching medical care*, excluding sepsis, 60% were due to CNS trauma. In recent conflicts involving U.S. forces, including Desert Storm, CNS trauma accounted for almost one-half of all battle-related casualties. In spite of this, no effective therapy exists for treating this devastating condition.

For U.S. society at large, traumatic injury to the brain and spinal cord is also a significant health problem. Traumatic brain injury (TBI) is the most common cause of traumatic death and disability. Approximately 52,000 patients in this country die each year as a direct consequence of TBI. This represents almost one-third of all injury related deaths. Brain injury occurs every 7 seconds and results in death every 5 minutes. The cost for direct medical care is estimated to be more than $4 billion per year. (Morgan, 1994; Sosin et al., 1995)

The financial burden imposed by TBI is staggering. There are an estimated 80,000 survivors from civilian injury each year who are left with residual neurologic deficit that results in loss of function despite extended rehabilitation. The estimated cost of this chronic treatment is approximately $60,000 per patient per year or $4.8 billion per year. Since most of these patients are less than 40 years old and are otherwise in good health, many will experience long term survival following the injury. Thus, over a patient's lifetime, the health care cost factor is a major consideration. Additionally, there is loss of the productivity of these patients during their primes of life. (Giacino and Zasler, 1995)

Even minor head injury can lead to significant disability. A study of patients who sustained relatively minor injuries revealed up to 79% had residual symptoms 3 months after injury. One-third of these patients were unable to return to their jobs. The estimated incidence of all kinds of head injuries (minor to major) is greater than 8 million per year. Thus one can appreciate why brain injury poses a major social, financial and emotional problem. (Rimel et al., 1981)

Traumatic injury to the brain exhibits two phases. The first is neuronal injury that occurs as a direct result of the initial traumatic event. The secondary or late phase occurs from multiple neuropathologic processes and can continue for days to weeks after the initial insult.(Casey and McIntosh, 1994)

The primary injury phase is immediate, and thus is not amenable to treatment. If injury is sufficiently severe, death can occur almost instantaneously. The damage that occurs from this primary phase is often complete before medical care can be instituted. The most effective way to treat this is prophylaxis, such as wearing a motorcycle helmet.

The secondary injury phase is delayed and can be prolonged. Thus, there is an opportunity for therapeutic intervention. Injury at this level is both neuronal and glial. It is thought that the majority of neurologic injury occurs after the primary insult and is thus related to this secondary injury. The processes that contribute to this "neuron suicide" include inflammation, effects of free radicals, excitatory amino acids (EAA), certain ions (e.g., Ca^{++}), and ischemia.(Chestnut and Marshall, 1993)

For temporal reasons, efforts to develop therapeutic approaches to TBI have focused on secondary injury processes. Although significant research efforts have been devoted to this issue, current clinical treatment is largely confined to supportive and palliative measures with particular emphasis on maintaining cerebral perfusion, minimizing intracranial hypertension and indirectly treating cerebral edema. Efforts to develop treatment modalities aimed at ameliorating secondary injury have been disappointing.

Since the 1960's, significant advancements have been made in treating neurotrauma. The focus has been on ameliorating the early phase of secondary injury. Improvements in cardiovascular and ventilatory management have contributed to these improvements. Intracranial pressure control, early hematoma evacuation, etc. are all important aspects of modern treatment. As a result, today, the brain injured patient has an over 80% probability of survival as opposed to only 20% before. A key element in this management is the recognition that early identification of the injury and rapid evacution to a neurotrauma center are essential. Only by doing so can the new management approaches be successful.

For the military, these approaches are not feasible. The leading problem, rapid evacuation , is not usually possible. Patients are often located at remote sites, which are not militarily secure. Even with air superiority, as in Desert Storm, evacuation time was over 45 minutes. Delays in treatment ranged from 2 to 24 hours. Application of treatments found in civilian emergency rooms are not practical in the far forward environment. The first responder is a combat medic with basic provider skills. The medic's primary missions are to provide hemodynamic stabilization and protect the patient. More advanced care is not reasonable in this hostile location.

For these reasons, the focus of relevant military research should be to ameliorate the later stages of secondary injury. Inflammation is a crucial component of this. Brain edema is the most clinically important effect of inflammation, as it is the primary cause of decompensation in these patients. For injuries sustained outside of the brain, it is well established that amelioration of edema using antiinflammatory therapies, ice and nonsteroidal antiinflammatory agents, will significantly hasten recovery and improve outcome (Farry et al., 1980; Cipolle et al., 1994). Prompt use of corticosteroids following spinal cord injury has been shown to improve neurologic outcome (Bracken et al., 1990, Bracken et al., 1992). However, steroid therapy for TBI has been disappointing

(Dearden et al., 1986). Why neuroinflammation treatment has not been effective is largely due to an inadequate understanding of the basic mechanisms regulating neuroinflammatory processes.

There are 3 hypotheses to our study. They are:

1. Interleukin-1β, TNF and IL-6 are important mediators of neuroinflammation.

2. The cellular inflammatory effect of each cytokine is specific.

3. The neuroinflammatory state initiated by a particular cytokine is sustained by the release of other cytokines.

There are two principle objectives for this proposal. The first is to identify the immunomodulators most likely responsible for initiating and maintaining the neuroinflammatory response. The second is to characterize the time effect sequence of these immuno-modulators. This will be done on an intact mammalian level.

Previously, we developed an *in vivo* model of neuroinflammation using uninjured canines. Although these animals are not injured, we are able to recreate in a noninvasive manner the release of cytokines into the CNS as would occur after trauma. Using this approach, we provided the first direct evidence that cytokines initiate the neuroinflammatory process. On an *in vivo* intact physiologic level, interleukin-1β (IL-1) leads to a rapid and highly specific lymphocytic leukocytosis in the cerebrospinal fluid (CSF). This effect occurs within 10 minutes of the appearance of IL-1 within the CNS and is concentration-dependent.

At this midpoint of our project, we have successfully made significant progress towards meeting our objectives. We continue to characterize the cytokine role in initiating the neuroinflammatory state. Our studies identify and characterize the cytokines sustaining the neuroinflammatory lymphocytic cellular responses. Using pharmacokinetic analysis, we determine the rate of IL-1 elimination from the CNS. This is used to delineate the contribution of IL-1 to the observed prolonged response. Finally, we demonstrate that tumor necrosis factor α (TNF) and not IL-1 is the likely cause of the sustained response.

A better understanding of neuroinflammation is being realized. Knowledge of the mechanisms by which the neuroinflammatory cascade is initiated and subsequently maintained offers the basis for a rational and effective therapy.

BODY

Experimental Methods

On the day of experiment, animals are sedated with pentobarbital (10-15 mg/kg, i.v.). Warming pads and blankets are used to maintain core body temperature. Animals are monitored continuously for airway protection but this dose of sedative has not compromised ventilation.

Baseline (pretreatment) cerebrospinal fluid (CSF) and blood samples are collected prior to any cytokine administration. CSF is removed via the Ommya reservoir using a 25 g. butterfly infusion set. Under sterile conditions, the needle is placed into the Ommaya reservoir, which is surgically placed in a subcutaneous site at the top of each animal's head (see Procedures). The distal end of the butterfly tubing is used to withdraw CSF using 1cc tuberculin syringes. A maximum of 300 μl is sampled.

Blood is removed from either a forelimb i.v. (canine) or via the previously implanted internal jugular vein catheter (swine). In the canine model, a 22 g. angiocatheter is placed under sterile conditions into a forelimb vein. This allows multiple sampling with minimal venipunctures. At the end of experiment, the i.v. is removed. In the swine, veins are not easily located. Therefore, a central venous catheter is surgically implanted (see Procedures). This is chronically maintained and allows both sedative drug administration and blood sampling.

Cytokines were initially purchased from Collaborative Labs, a subsidiary of Becton-Dickenson. However, we discovered that Collaborative Labs is a distributor for R&D Systems, Inc. (Minn, MN), who is the primary producer. We now purchase the cytokines directly from R&D Systems. Cytokines come carrier free. They are suspended in sterile 0.1%BSA in PBS for canine experiments or 0.01% PSA (pig serum albumin) in PBS for swine experiments. IL-1 is then administered into the CNS via the Ommaya reservoir ventricular shunt system described earlier. No more than 0.5 cc volume is given so as to minimize changes in intraventricular fluid dynamics and intracranial pressure. Serial CSF and blood samples are obtained over the next 24 hours.

Doses in canines range from 50 to 200 ng/kg, which is comparable to cytokine concentrations observed in injured rat models and human patients (McClain et al., 1987; Goodman et al., 1990; McClain et al., 1991; Taupin et al., 1993; Shohami et al., 1994; Fan et al., 1995; Yamasaki et al., 1995). In swine, doses are given as mg/animal. The purpose for expressing doses in this way is that there can be a wide range of body weight for pigs (20-50kg) without a significant change in brain size. Because brain weight is equivalent between canine and swine, dose ranges administered as total cytokine received per animal is equivalent between these two species. Control animals receive an equivalent volume of sterile o.1% BSA in PBS or 0.01%PSA in PBS.

Within 10 minutes of collection, plasma is separated from other blood constituents. Plasma samples are then placed at -70°C for storage. CSF is placed at -70°C immediately after collection.

For experiments measuring body temperature, no sedation is required. As there is no biofluid sampling, there is minimal subject discomfort. Temperature is measured continuously using a rectal probe and a digital thermometer (Mallinkrodt, Inc, St. Louis, MO).

Analytical Methods

Cell counts are determined in whole blood and CSF samples using an automated hematology analyzer (Cell-Dyn 500, Abbott Laboratories, Inc, Chicago, IL). We adapted this device to CSF use (Appendix I). Specimens were analyzed within 5 minutes after collection. This was to minimize any WBC deterioration that is known to occur in CSF that is left standing for more than 30 minutes.

This automated hematology analyzer uses a flow cytometry-based approach employing a xenon laser rather than electrical impedance. In brief, cells flow across an aperature. A laser beam is pulsed through the aperature allowing each white blood cell to be counted when it breaks the beam as it flows by. The scattering of the laser beam across the nucleus allows each cell to be differentiated based on nuclear morphology. Traditional manual methods of cell sorting requires counting and identifying each cell. As it is impractical for anyone to count over 100 cells in multiple specimens in a meaningful time, only a sample high powered field is acutally examined. From this, total counts are extrapolated. As this analyzer counts and speciates every leukocyte, results are more obtined more rapidly and accurately. Analysis time for a 125µl specimen is approximately 30 seconds.

Cytokine concentration in both CSF and plama are determined using a double antibody electrochemilumenescence assay. Standard concentrations of cytokine are prepared from cytokines purchased from R&D Systems, Inc. (Minn,MN). In brief, this assay uses two antibodies. One is a monoclonal mouse antibody and the other is a polyclonal goat antibody. Both are directed against different epitopes on the cytokines being studied. One antibody is attached to ruthenium, a light emitter. The other is biotinylated. Separation of bound from free antigen is accomplished with streptavidan beads. Emitted light is detected with an Origen analyzer (Igen, Gaithersburg, MD). This assay has a standard curve ranging from 2000 to 7 picogms/ml.

Dose-response curves are constructed on a log-linear plot using best fit linear regression analysis. The leukocyte count at each dose is compared to control using student's t-test. Time-effect profiles are fit by nonlinear regression analysis (Table2D Curve, Jansen, San Rafael, CA). Area under the curve

comparisons between each dose and control uses the nonparametric Mann-Whitney U test. Individual data points are expressed as the mean ± standard error of the mean (SEM). Comparison between individual points are by t-test and among multiple points by analysis of variance. Significance was defined before study as $p \leq 0.05$.

Pharmacokinetic analysis is performed using a commercially available curve-fitting program (Table2D Curve, Jansen, San Rafael, CA). Each animal is analyzed individually. The data is fit to a 2 compartment model of elimination. The method of residuals is applied to determine the alpha distribution phase. Pharmacokinetic results of elimination constant (k), half life (T1/2), volume of distribution (Vd) and clearance (Clr) are determined for both the alpha (α) and (β) phase. The beta phase is by convention reflective of the central (c) compartment in the 2 compartment model of elimination. Results are given as the mean ± SEM.(Gibaldi and Perrier, 1973)

Assumptions

The first assumption is that there is sufficient homology between canine, porcine and human cytokines allowing use of human cytokines in these animal species. If homologous, the observed effects are due to intrinsic cytokine properties and are not foreign protein reactions. There is a high level of preserved homology among higher animal species. Previous literature notes over 80% homology (ref). Also, our work demonstrates a predominantly lymphocytic response to IL-1. Generally, in foreign protein responses, the predominant leukocytes are neutrophils. Thus, it is unlikely that the response is due to the few nonhomologous regions.

The second assumption is that there is sufficient homology between canine, porcine and human cytokines so that using an analytical method based on antibodies directed against human cytokines will also recognize canine and porcine produced cytokines. This is assured by our finding that this method is able to measure TNF that is produced by each animal.

The third assumption is that canine and swine are representative of the human condition. Previous immunologic studies have shown that the 2 animal species have similar systemic immunologic properties as humans (ref). These subjects are the highest order animal species that could be reasonably used for these studies.

Procedures

Canine

Male beagle dogs weighing between 10 - 15 kg are surgically prepared with an indwelling intracerebroventricular (i.c.v.) catheter. All surgeries use strict sterile procedures and enflurane/oxygen anesthesia. Preoperatively, dogs receive dexamethasone, 2 mg, i.m. the night before surgery.

This i.c.v. catheter implantation is a modified method initially developed for rhesus monkeys (Poceta et al., 1981; McCully et al., 1990). After reflection of skin, fascia and overlying temporalis muscle, a 1/8 inch diameter burr hole is made though the skull 1 cm lateral to the mid sagittal suture and 3 cm posterior from the orbital ridge. The dura is pierced with a 20 g. needle but the underlying parenchyma is not violated. A Pudenz catheter is advanced through the burr hole into the frontal horn of the ipsilateral lateral cerebral ventricle. The depth is approximately 4 cm. Placement is confirmed by the return of clear CSF. The distal end of the catheter is attached to a modified, flat-bottomed Ommaya reservoir. The reservoir is sutured to the periosteum against the outer table of the calvarium. The overlying tissue is closed in layers with suture.

Postoperatively, wounds are treated topically with Bacitracin for 3 days. Dogs receive dexamethasone, 2 mg, i.m., bid, for 3 days and cephazolin, 0.25 gms, i.v., bid for 5 days. Perioperative discomfort is treated with buprenorphine.

Swine

Male Yorkshire pigs weighing between 20 - 30 kg are surgically prepared with an indwelling intracerebroventricular catheter and jugular vein catheter. All surgeries use strict sterile procedures and enflurane/oxygen anesthesia.

This i.c.v. catheter implantation is a modified method of that described above. As before, a 1/8 inch diameter burr hole is made though the skull. However, for proper catheter placement, the burr hole site is located 1 cm lateral to the mid sagittal suture and 1 cm posterior from a line connecting the posterior canthuses of the eyes. The dura is pierced with a 20 g. needle but the underlying parenchyma is not violated. A Pudenz catheter is advanced through the burr hole into the frontal horn of the ipsilateral lateral cerebral ventricle. The depth is approximately 4 cm. Placement is confirmed by the return of clear CSF. The distal end of the catheter is attached to a modified, flat-bottomed Ommaya reservoir. The reservoir is left lying against the outer table of the calvarium. The overlying skin is closed with suture.

A venous catheter (Tygon, 0.04"ID x 0.07"OD) is placed into the jugular vein by a modified method described by Terris et al. (1986). The catheter was placed into the internal jugular vein and advanced to but not into the right atrium. The distal end is subcutaneously tunneled to the back of the pig's neck. There it

is exteriorized and sutured to the skin. A needleless hub is attached and the catheter is filled with heparinized saline (1000 units/ml).

Postoperatively, wounds are treated topically with Bacitracin for 3 days. Pigs receive dexamethasone, 2 mg, i.m., qD, for 3 days and cephazolin, 1 gm, i.v., bid for 5 days. Perioperative discomfort was treated with buprenorphine, prn. All catheters are flushed daily for 1 week. The i.c.v. catheter/Ommaya reservoir is flushed with sterile saline. The i.v. catheter is flushed with heparinized saline (50units/cc). After the first week, the i.v. catheter is flushed once per week but the i.c.v. catheter is not flushed. Animals are allowed to recover for 4 weeks before any experiments.

A post-operative period of at least 3-4 weeks is allowed prior to any experiment. This allows complete wound healing and thus re-establishment of the integrity of the blood-brain barrier.

Each animal is allowed at least 1 week between experiments to ensure resolution of any residual cytokine effect. As no injury occurs during the experiment, the immunologic response to a given cytokine should be complete by that time. This allows additional experiments in the same subject.

Results

In canines, after a single i.c.v. administration of IL-1β, there is a dose-related increasing CSF leukocytosis. This occurs rapidly after administration and reaches statistical significance within 10 minutes (Fig.1). The minimally effective IL-1 dose range is 50 to 200 ng/kg. Within 10 min. after administration of the 100 and 200 ng/kg doses, the white blood cell counts are 36 cell/mm^3 ± 8.7 (mean ± SEM) and 102 cells/mm^3 ± 39.3, respectively. These results are significantly different from control, 9.3 cells/mm^3 ± 9.3 ($p<0.05$, t-test). At 50 ng/kg, the cell count is 12 cells/mm^3, which is not significantly different from control.

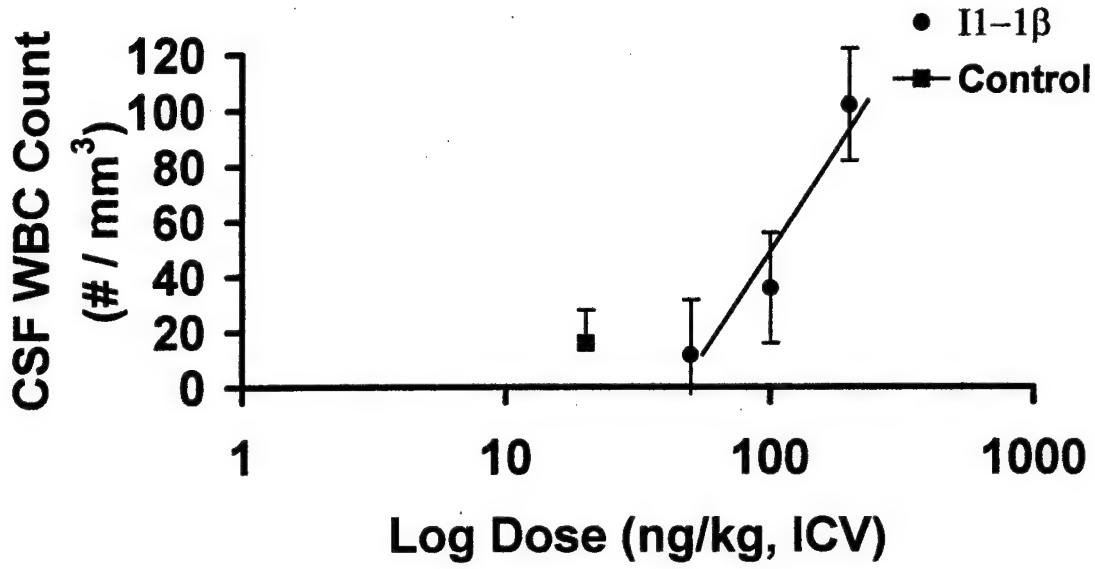

Fig 1: Dose response curve for IL-1 induced CSF leukocytosis in canines. Each point represents the total WBC per mm^3 count (mean ± SEM). The (●) are IL-1 treated and (■) control.

Preliminary results in swine reveal a similar dose-response relationship. Following a 2μg/pig, i.c.v. IL-1 dose, there is a CSF leukocytosis of 34 cell/mm^3 at 5 minutes after dosing and after 3μg/pig, i.c.v., 58 cell/mm^3. There is no evidence of CSF leukocytosis in pigs receiving vehicle control (Fig 2).

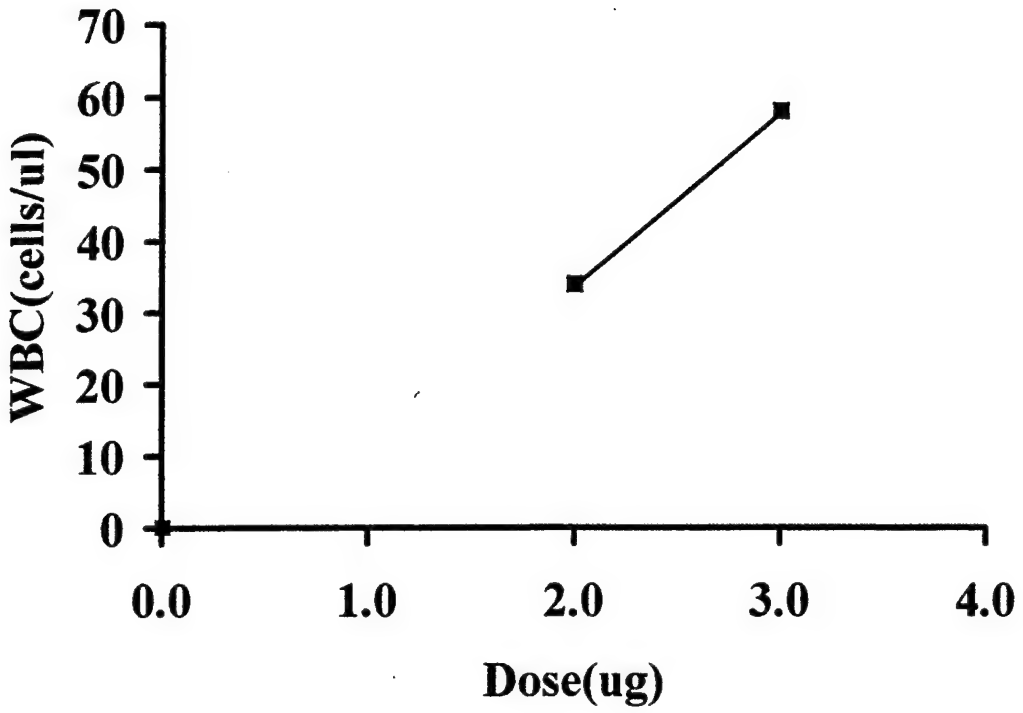

Fig 2: Dose response curve for IL-1 induced CSF leukocytosis in swine. Each point represents the total WBC per mm^3 (mean).

At the highest dose tested in canines, 200 ng/kg, there is a sustained CSF leukocytosis. This peaks at 20 minutes and then decreases. At 1 hour, there is a slight increase to 63 cells/mm^3 which remains sustained (Fig. 3). At lower doses, after the initial peak, there is a rapid return to baseline. Thoughout, this was significantly greater than control. For both 50 and 100 ng/kg IL-1 doses, by 15 minutes, WBC levels are not significantly different from control.

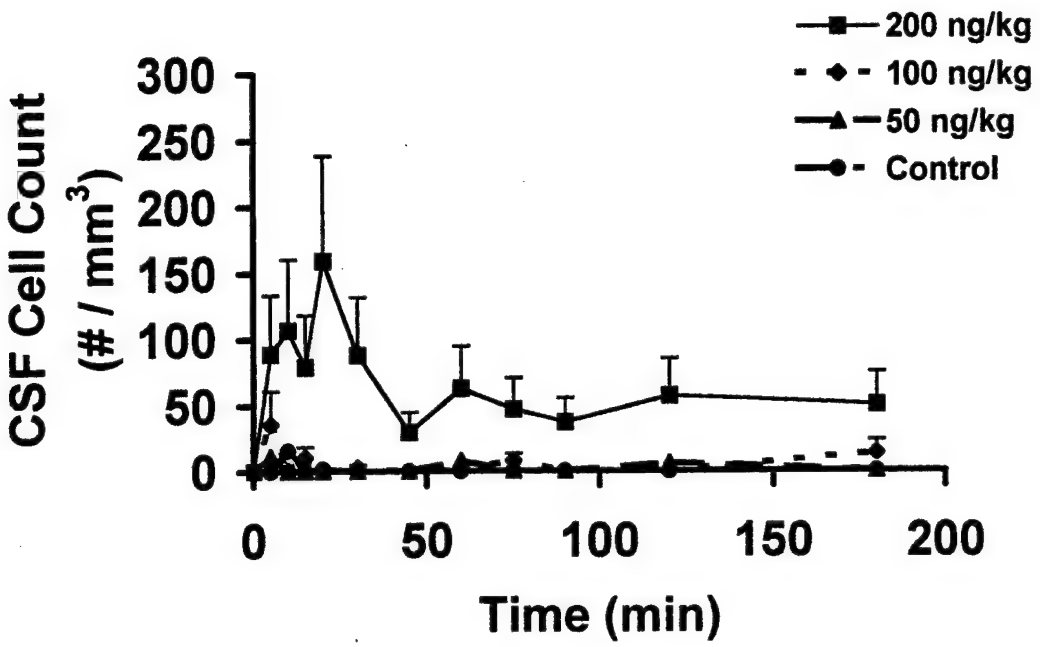

Fig 3. Time-effect for IL-1 induced CSF leukocytosis in canines. Each point represents the number of WBC per mm^3 of CSF as mean + SEM. The (■) are for the 200 ng/kg dose, (◆) 100 ng/kg, (▲) 50 ng/kg and (•) control.

Preliminary results in the swine reveal a significantly less sustained response (Fig. 4). After initial leukocytosis, the effect quickly waned so that by 15 minutes after dosing, the levels return to baseline. Higher doses need testing. It is also apparent from the peak response that the 3μg/pig dose is not equivalent to the 200 ng/kg canine dose. There was no evidence of a persistent CSF leukocytosis in pigs receiving vehicle control.

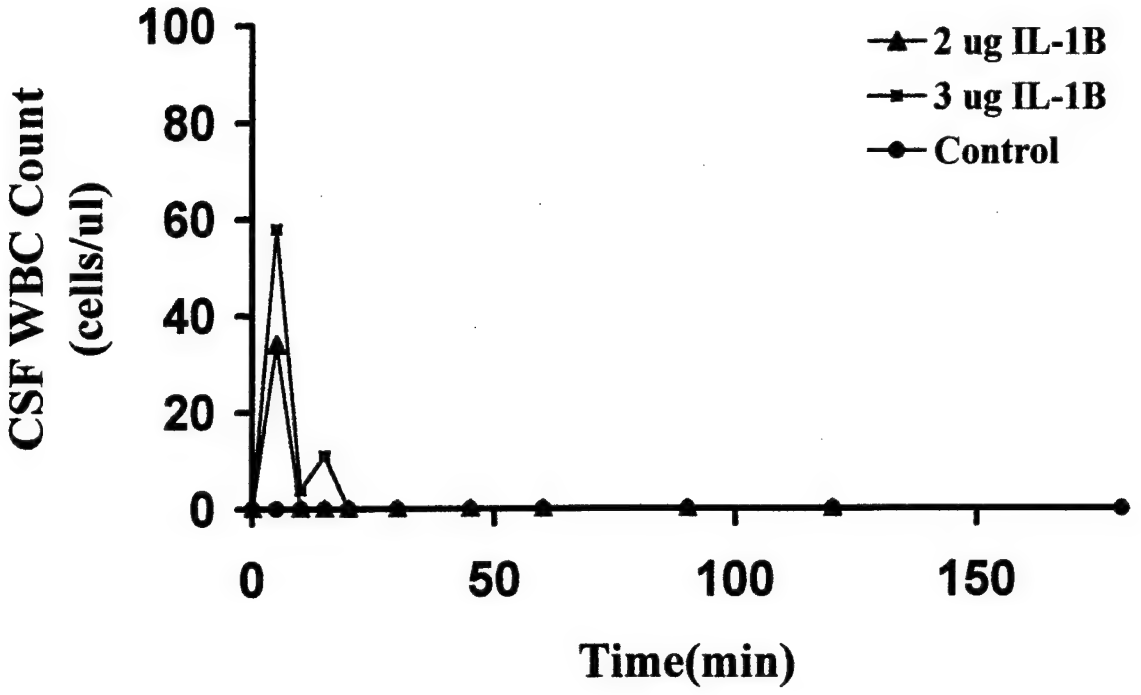

Fig 4. Time-effect for IL-1 induced CSF leukocytosis in swine. Each point is the number of WBC per mm^3 of CSF. The (▲) represent the 2 μg/pig dose, (■) 3 μg/kg and (•) control.

Throughout the study period, the canine white cells were noted to be mononuclear (lymphocytes and monocytes), approximately 75% of the total white cell population (Fig. 5). The predominant species was identified as lymphocytes, which ranged from 50 to 77%. At 180 minutes, there was a transient shift to neutropils, 55 % vs 27% lymphocytes. This needs further investigation.

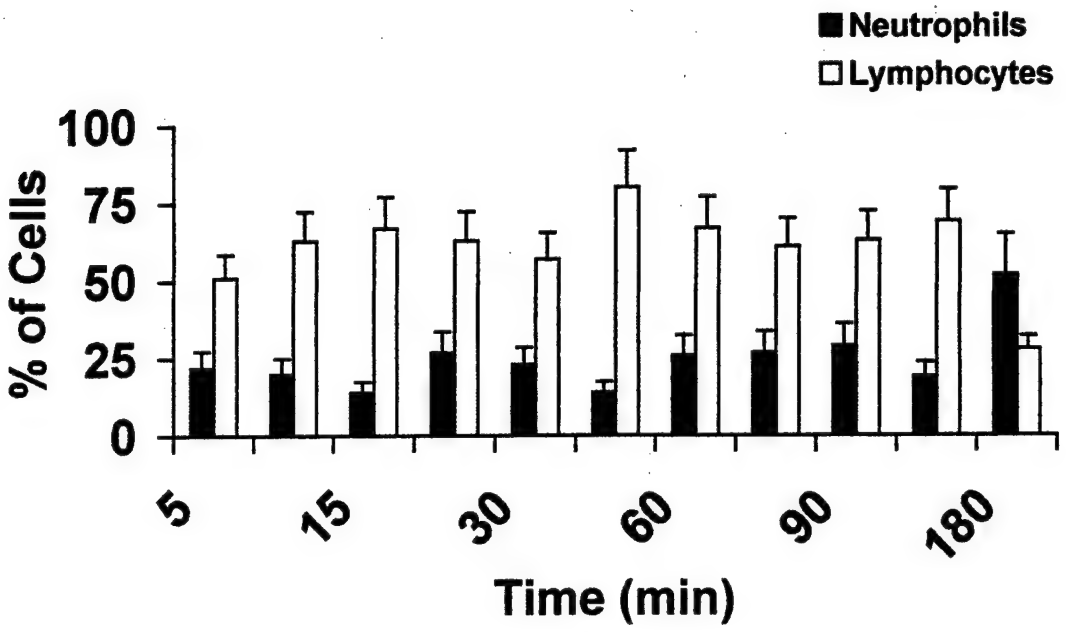

Fig. 5. Time-effect of IL-1 induced lymphocytosis and polymorphonucleosis in canine. Each bar is representative of the mean percentage of the total cells. The (□) is the lymphocytes and the (■) is the polymorphonuclear cells.

The response in swine is also predominantly lymphocytic. (Fig 6).

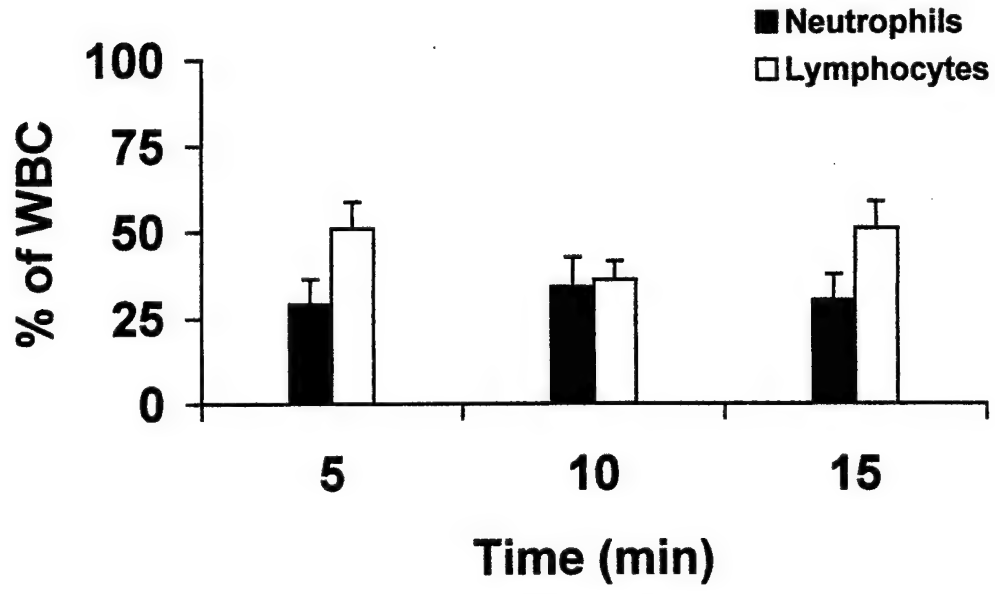

Fig. 6. Time-effect of IL-1 induced lymphocytosis and polymorphonucleosis in canine. Each bar is representative of the percentage of the total cells (mean ± SEM). The (□) is the lymphocytes and the (■) is the polymorphonuclear cells.

Over the same period, in both canines (Fig. 7) and swine (Fig. 8), there are no significant changes in leukocyte in peripheral blood. There is also no significant difference in canine body temperature in the 200 ng/kg, i.c.v. treated group when compared to control (Fig. 9).

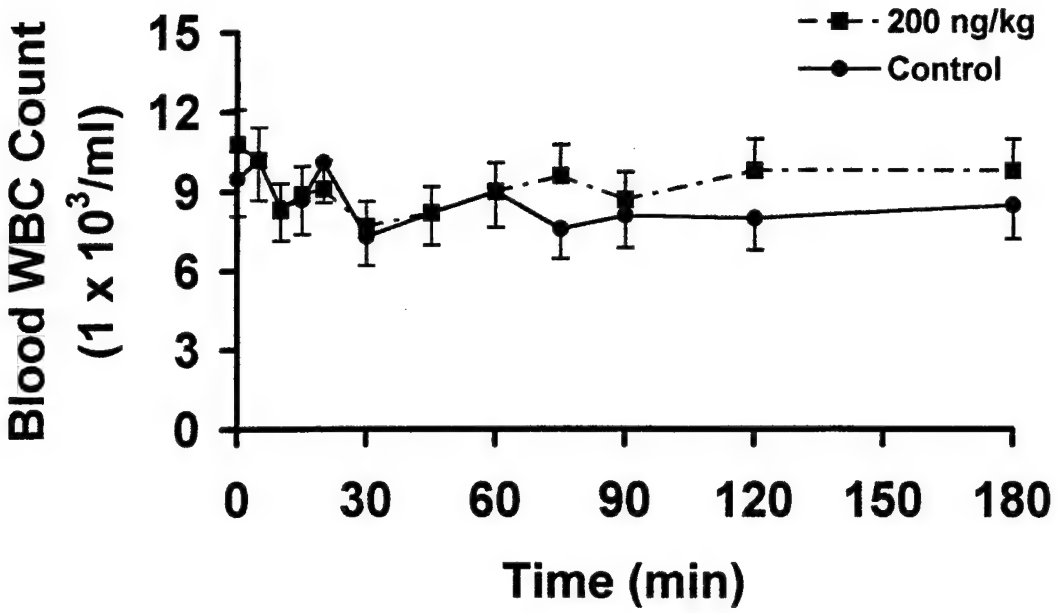

Fig. 7. Time-effect of IL-1 induced leukocytosis in canine blood. Each point represents the mean ± SEM. The (■) is the 200 ng/kg, i.c.v. and (•) control.

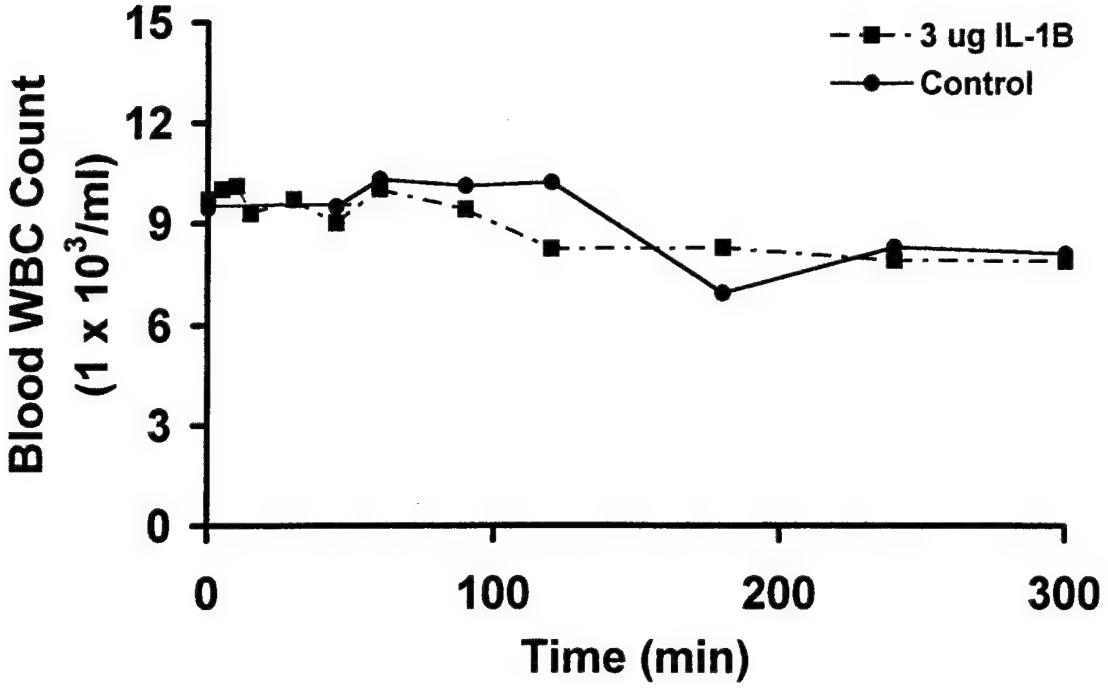

Fig. 8. Time-effect of IL-1 induced leukocytosis in swine blood. The (■) is 3 μg, i.c.v. and (•) control.

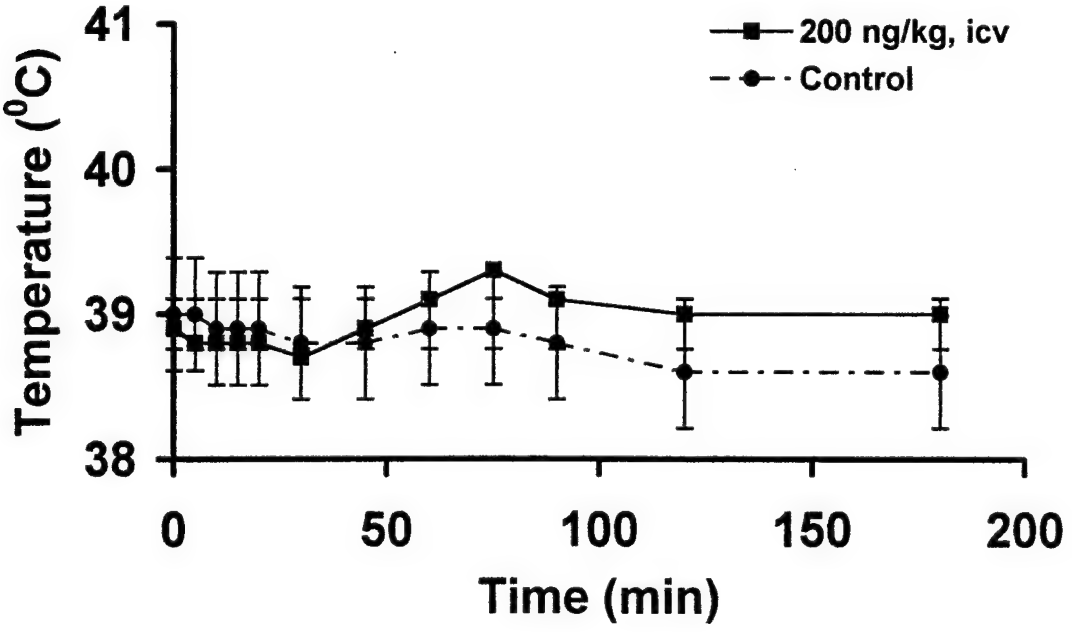

Fig. 9. Time-effect profile of IL-1 induced core body temperature changes after i.c.v administration. Each points represent the mean ± SEM. The (■) is the 200 ng/kg, i.c.v. and (•) control.

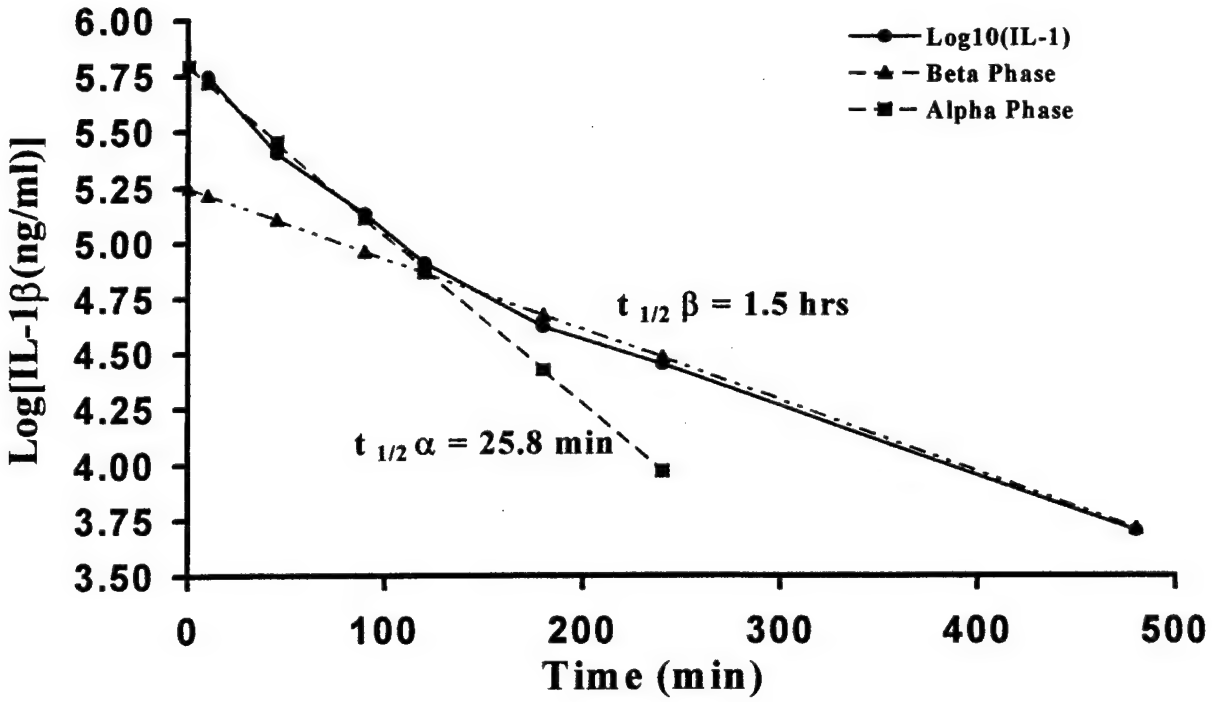

Fig. 10. CSF IL-1 elimination profile of a representative canine subject on a log-linear scale. Each (•) is the log concentration of IL-1 in CSF. Each (▲) represents the log concentration of IL-1 in the beta phase or central compartment. Each (■) is the log concentration of IL-1 in the alpha or peripheral compartment.

Pharmacokinetic analysis of IL-1 in the CNS reveals a two phase elimination profile (Fig. 10). The data fits a 2 compartment open model of elimination. Figure 11 shows the elimination of a representative animal. The initial distribution phase has an elimination T1/2 of 20 minutes $\pm$ 1.5 (SEM). The terminal elimination phase has a T1/2 of 2.2 hours $\pm$ 0.2 (SEM). The volume of distribution for the central compartment is 4.9 cc $\pm$ 0.5 (SEM). Thus the clearance rate for IL-1 is 0.09 cc/min.+ 0.008 (SEM) (Table 1).

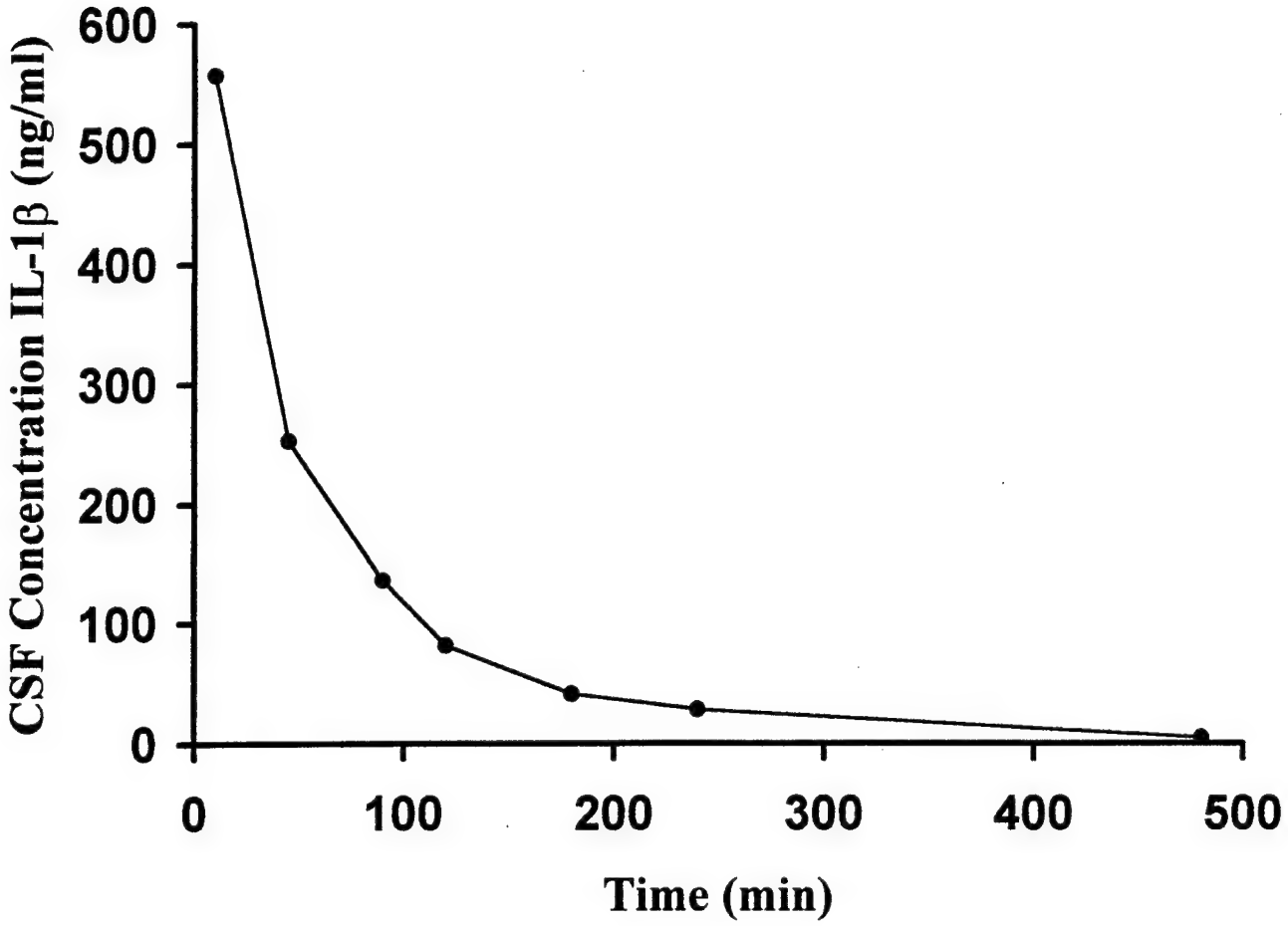

Fig. 11. CSF IL-1 elimination profile of a representative canine subject on a linear scale. Each (•)is the concentration of IL-1 in CSF.

	Alpha:			Beta:			
	t 1/2 (hr)	K_α (1/hr)		t 1/2 (hr)	K_β (1/hr)	aVd_c(cc)	Clr_c(cc/min)
AVG	0.33	2.86	AVG	2.20	0.47	4.91	0.09
STE	0.03	0.26	STE	0.21	0.04	0.53	0.01

Table 1. Pharmacokinetic parameters of IL-1 elimination from canine CSF.

Preliminary pharmacokinetic study in swine reveals a terminal T1/2 of 1.4 hours. The volume of distribution for the central compartment is 15 cc's with a clearance rate of 0.13 cc/minute (Table 2). Figure 12 shows the elimination of IL-1 from CSF in a representative subject pig. This data also fits a 2 compartment open model of elimination (Fig. 13).

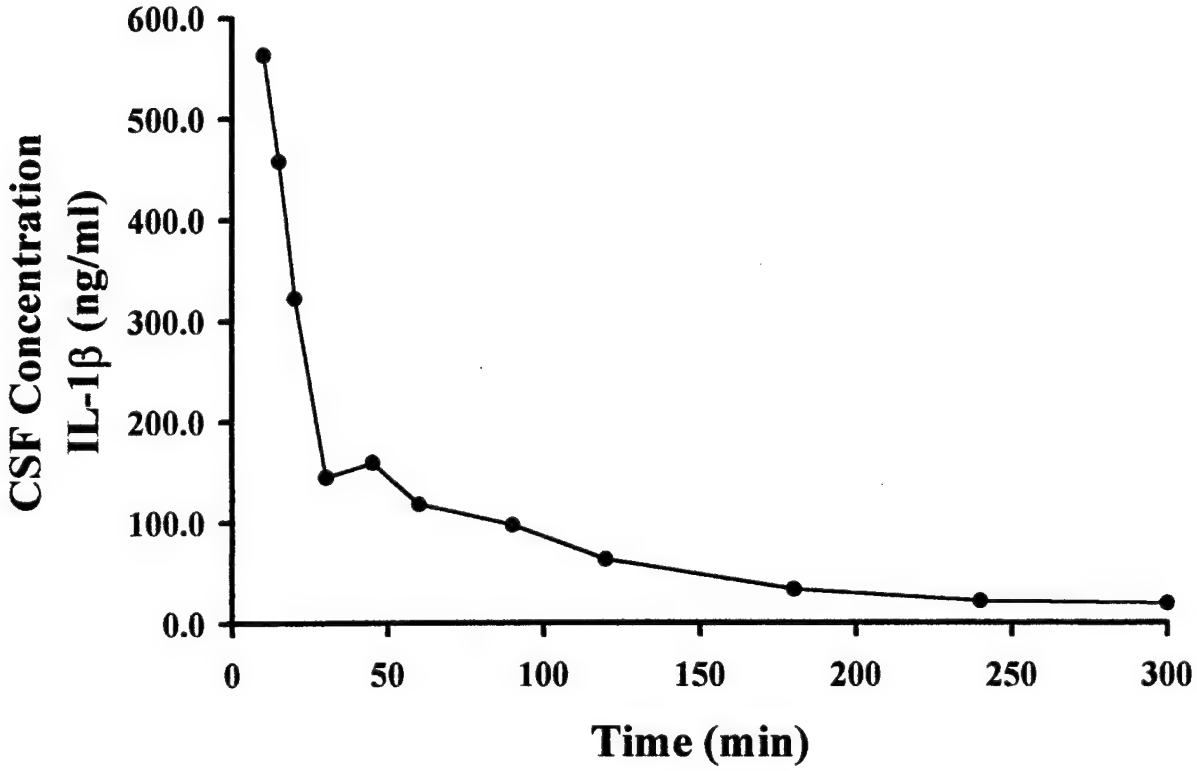

Fig. 12. CSF IL-1 elimination profile of a representative swine subject on a linear scale. Each (•)is the concentration of IL-1 in CSF.

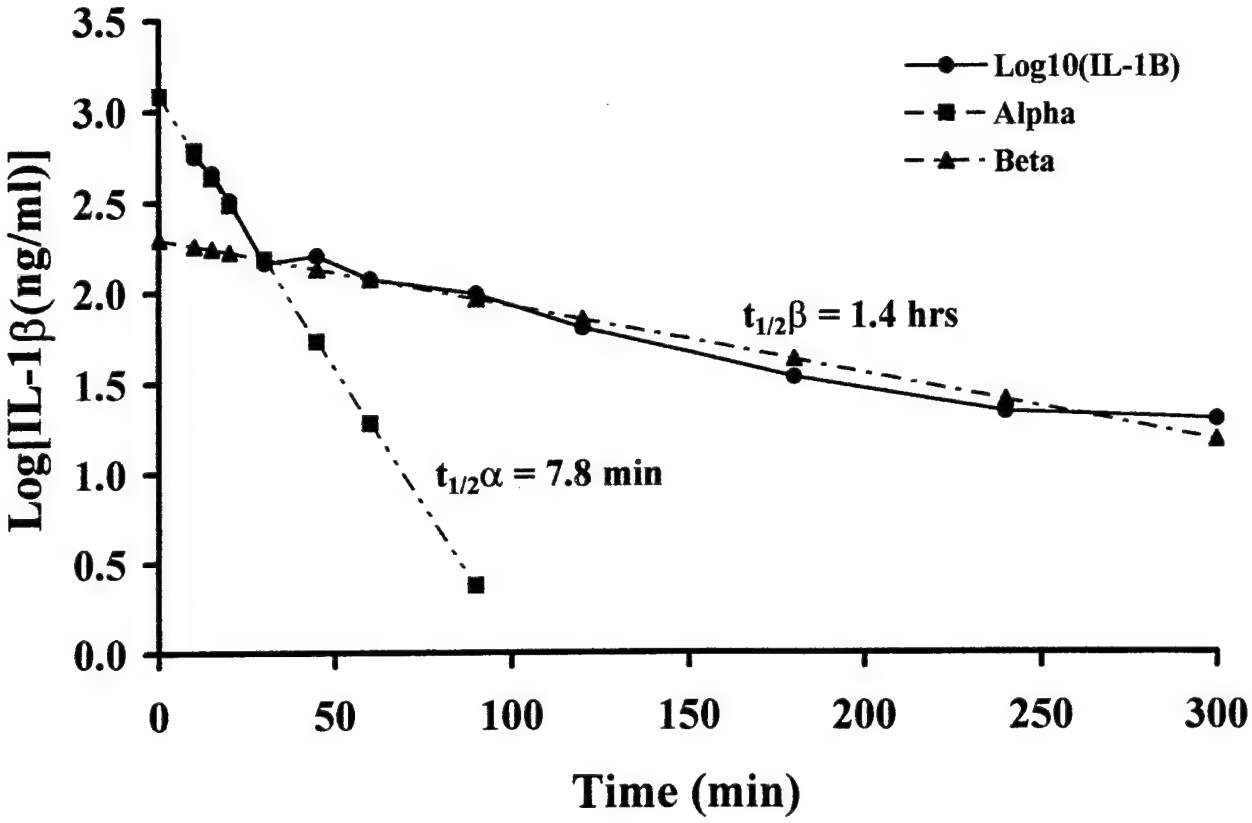

Fig. 13. CSF IL-1 elimination profile of a representative swine subject on a log-linear scale. Each (•) is the log concentration of IL-1 in CSF. Each (▲) represents the log concentration of IL-1 in the beta phase or central compartment. Each (■) is the log concentration of IL-1 in the alpha or peripheral compartment.

	Alpha:		Beta:			
Exp	$t_{1/2}$ (hr)	K_α (1/hr)	$t_{1/2}$ (hr)	K_β (1/hr)	aVd_c (cc)	Clr_c (cc/min)
1-45	0.13	5.34	1.35	0.51	15.21	0.13

Table 2. Pharmacokinetic parameters of IL-1 elimination from swine CSF.

A group of canines is tested to ascertain whether or not IL-1 challenge induces endogenous production of other proinflammatory cytokines, i.e., TNF and IL-6. In Figure 14, it is apparant that TNF is rapidly produced in canines. It rises above baseline at 30 minutes to a peak response at 2 hours and then

decreases. However, there is no evidence for IL-6 production in the CNS. Furthermore, TNF and IL-6 are not detected in plasma.

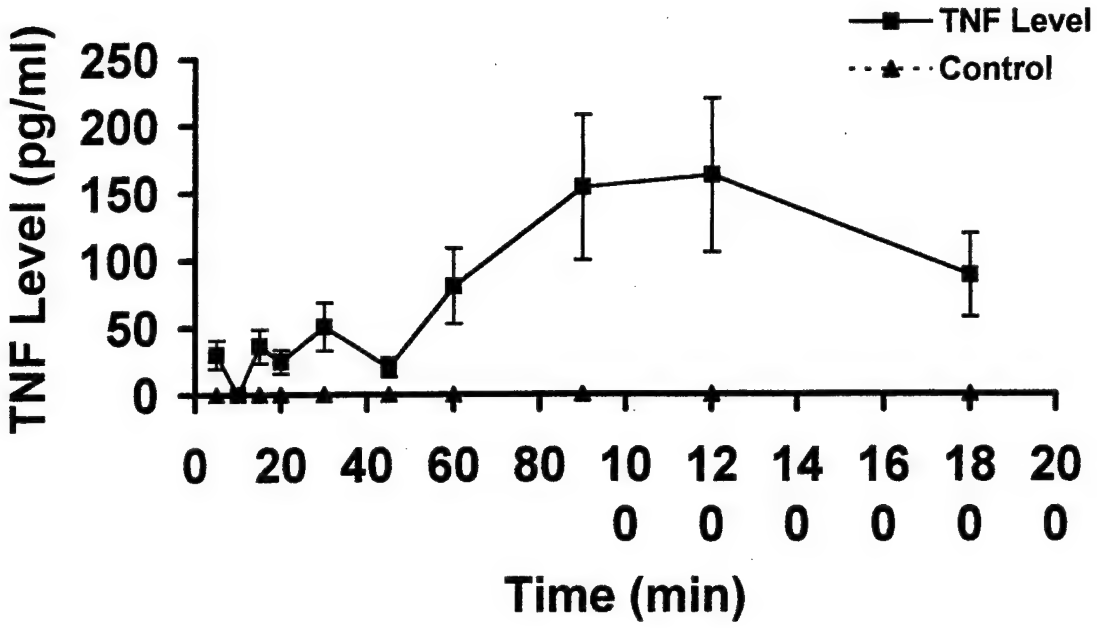

Figure 14. TNF levels in CSF after IL-1 administration. Each point represents the mean ± SEM. The (■) is the 200 ng/kg dose group and the (▲) control.

Preliminary study in swine reveals no production of TNF. This may be a function of IL-1 dose as the inflammatory state is not sustained at 3 μg/pig, the highest dose tested so far.

Discussion

Using a unique novel model of neuroinflammation, we are able to study on an intact physiologically relevant level, processes regulating inflammation in the CNS. This model is unique in that it allows access to the CNS without acute disruption of the blood-brain barrier. It is well recognized that traumatic injury leads to a cascade of biochemical events culminating in secondary injury. Part of that sequence is the release of inflammatory mediators. We believe that the most important of these are the proinflammatory cytokines, IL-1, TNF and IL-6. Our model, which uses a healthy noninjury state, allows us to recreate the release of these cytokines but in an experimentally controlled fashion. This allows us to identify the roles played by each.

It is crucial that the integrity of the blood-brain barrier be maintained. The CNS is unique because of the blood-brain barrier. This anatomic structure regulates passage of all material, e.g., ions, cells, proteins, etc., into and out of the CNS. By doing so, it provides an important additional layer of protection that does not exist for other organs. If this barrier is disrupted, e.g., by acute needle puncture, then cells, foreign particles and immunoactive substances can freely enter the brain. Also, the blood-brain barrier is living tissue. After a needle puncture, it will give rise to its own immunologic reaction. Thus to appropriately explore physiologically relevant mechanisms by which the CNS regulates the neuroinflammatory response, the blood-brain barrier must remain intact.

In the work thus far, we have made significant progress toward meeting both objectives. IL-1 mediates a dose-dependent neuroinflammatory response in the CNS. By using leukocytes as a marker of inflammation (as it is widely used in clinical medicine), we show that IL-1 produces a predominantly lymphocytic leukocytosis.

The IL-1 lymphocytosis is long lasting, greater than 3 hours. This is surprising as the inflammatory effect of IL-1 in the periphery, following single dose administration, is very rapid (Kampschmidt and Jones, 1985). The reason for the fast resolution of IL-1 induced cellular inflammation in the periphery is the rapid rate of elimination of this cytokine from circulation. The mechanisms of this elimination from circulation is proteolysis and tissue distribution and are completed within a few minutes (Kampschmidt and Jones, 1985; Newton et al., 1988). Thus, a possible explanation for the prolonged IL-1 inflammatory effect in brain may be that IL-1 is more slowly eliminated from the CNS.

To address the issue of IL-1 elimination from the CNS, we report the first pharmacokinetic analysis of this cytokine in CSF. Our detailed pharmacokinetic analysis reveals that IL-1 has a terminal T1/2 of 2 hours. This is much longer than previously suspected. With this slow rate of elimination, it is possible that this accounts for the long duration of inflammation.

Examination of the time-effect profile of IL-1 reveals a peak leukocyte effect at 20 minutes followed by a rapid decrease to a sustained level. There is a small secondary peak at 1 hour but this too is transient. If the decline in leukocyte cell counts was due solely to the decline in IL-1 CSF levels, the cellular effect profile would more closely match that of IL-1 elimination, i.e., slowly decline over 2 hours. Furthermore, the IL-1 levels at 1 hour after 200 ng/kg are approximately 250 ng/ml. Similar CSF concentrations of IL-1 after lower doses (100 ng/kg and 50 ng/kg) are not associated with cellular effect. Thus, another explanation is needed.

Another possible explanation for IL-1's prolonged effect is the release of other proinflammatory cytokines. In the periphery, it has been shown that IL-1 can lead to the release of TNF and IL-6 (Abbas et al, 1997a; Abbott et al., 1991; Judd and MacLeod, 1995). However, as the CNS is considered immunologically privileged, this was thought not to be possible in the brain. In this study, we demonstrate that TNF is released following IL-1 administration. The source of TNF is uncertain but is clearly endogenously produced. *In vitro* sudies have shown that cultured astrocytes and microglia may have immunocompetence and can express these cytokines under appropriate conditions (Wong et al., 1984; Fontana et al., 1984; Vass et al., 1986; Massa and ter Meulen, 1987; Steit and Kreutzberg, 1988; Frei and Fontana, 1989; Giulian et al., 1989; Streit et al., 1989; Woodroofe et al., 1991; Colton et al., 1992; Brenner et al., 1993). Our study is the first to show that TNF can be produced *in vivo* on a fully intact physiologic level.

TNF begins to manifest 30 minutes after IL-1 administration. This time period is sufficient for RNA translation to occur. Thus, our work suggests TNF does not come from readily available stores but is produced in response to IL-1. Cytokine production is a feature of specific immune responses.

TNF peaks at 2 hours after IL-1 and then slowly decreases. This coincides with the continued leukocytic response noted in the CSF. Thus a more likely basis for the persistent inflammatory state seen after IL-1 is a TNF contribution. IL-6 does not appear to be involved.

It is interesting that IL-1 elimination is more prolonged in the CNS. One possibility is that IL-1 leads to its own production, i.e., autoproduction. There is evidence that IL-1 leads to autoproduction in the periphery (Abbas et al., 1997a). The data we provide cannot definitively rule-out IL-1 autoproduction. This is better demonstrated studying clearance of a known quantity of radioactive labelled IL-1 from the CNS. However, there are 2 features of our work that suggest that IL-1 is not autoproduced. The first is that the volume of distribution for the central compartment is approximately 5cc. This is approximately equivalent to the anatomical volume of the canine lateral cerebral ventricles. If there is autoproduction, the volume of distribution of the central compartment would be much smaller. The second is that there is no obvious absorption component to the elimination profile.

Another reason is that proteolysis may not play as prominant a role in IL-1 elimination from the CNS as it does in the systemic circulation. The rate of resorption of CSF from the arachnoid granulations in the CNS is about 20 cc/hr. The total CSF volume throughout the canine neuroaxis (brain and spinal cord) is approximately 30 - 40 cc. Thus, IL-1 should be cleared from CSF in about 2 hours. As IL-1 T1/2 is also about 2 hours, CSF turnover is the most likely primary source of elimination from the CNS.

In summary, our work demonstrates that IL-1 leads to a specific and prolonged inflammatory state. The basis of the persistent effect can be ascribed to IL-1 stimulating TNF reproduction, which in turn is the basis for delayed inflammation. From a clinical standpoint, this is very important. If a patient reaches medical treatment within 1 hour, then therapies directed against IL-1 have the potential for amelioration. However, if the evacuation is delayed (as in combat), then a more rational therapy would be against TNF.

Interestingly, in spite of the robust neuroinflammatory response, there was no evidence of fever at any of the doses tested. Fever is known to be an intrinsic property of IL-1. IL-1 was initially known as pyrogen. The lack of response suggests that this physiologic effect is either mediated by a peripheral mechanism, e.g., prostaglandin release, or perhaps higher than inflammatory doses are needed. Clinically, in autoimmune disorders where there is a generalized systemic inflammation, no fever is encountered. Also, in certain diseases characterized by CSF lymphocytosis such as viral meningitis, fever is not a prominent feature. IL-1 is well accepted as a fever producing cytokine and that it is thought to be mediated at the CNS level. As our findings raise intriguing challenges to dogma, further study is needed.

To our knowledge, this is the first study of its kind that identifies and characterizes the neuroinflammatory effect of a putative endogenous modulator. This work is particularly relevant scientifically and clinically because it is performed on a physiologically intact level and measured a widely accepted clinical marker of inflammation, i.e. leukocytosis.

We still have significant work to accomplish. Although preliminary results are promising, the transition to a swine model needs to be completed. We need to determine which cytokine mediates neutrophilic leukocytosis in the CNS. Previously we thought it would be TNF but a more likely candidate is IL-6. These experiments will be undertaken over the final funding period.

Problems

Swine Model

Attempts to replace the canine model with a swine model has been problematic. To date, we have been unable to maintain our chronic pig model for

an adequate study period. The problem is the prevalence of swine illnesses. These diseases are fairly common in farms, breeding facilities and laboratory animal departments. These are largely ignored by many investigators as these illnesses usually do not impact on their experiments. Either infection does not adversely effect their protocols or the animals are used only in acute studies so infections are not permitted adequate time to develop. Because of the need for blood brain barrier healing after surgery, we must maintain pigs chronically. Additionally, however, as we are investigating inflammatory processes, any systemic illness contaminates our findings. Many of these illnesses present insidiously (i.e., greater than 6 weeks) and can lead to sudden death in an otherwise apparently healthy animal. As we plan on maintaining a chronic model, this presents another problem. So far, we used 3 different species of swine and 4 different vendors (including the NIH breeding facilities). Our university is currently sanitizing the entire facility as there has been an outbreak of Erisipelothrix rhusiopathiae, a swine disease that causes sudden cardiac death. The source of the infection has been traced to pigs from vendors being used by other investigators.

Because of size and growth considerations, we initially planned to use the micro pig. The Panepinto micro pig is equivalent in size to a medium sized dog and does not grow at the rapid rate that other pig species grow. However, these animals are highly suseptible to disease and cannot regulate their body temperature well. We then attempted using the Yucatan mini pig. These animals have poor cardiovascular function and are suseptible to sudden cardiac death.

We are now using the Yorkshire breed, which has a reputation as the hardiest. Our colony is kept in their own room isolated from all animals, including pigs. The vendor is among the most expensive but is also one that has the reputation of being the most meticulous.

If this is unsuccessful, then we must conclude that this animal species is not appropriate. Should that occur, we will return to the noninjured canine model.

Recommendations

The use of swine was previously proposed in the initial grant application. Difficulties in developing this model are enumerated above. In order to be certain that progress is made towards the study objectives while these difficulties are being addressed, experiments should be conducted in parallel using canines.

The uninjured canine model was previously demonstrated by us to be a reliable and reproducible model. In addition, the canine model uses the least number of animals and is associated with the least amount of surgical manipulation and overall discomfort to the subjects. In fact, the previous dog colony reached 2 years of study (the maximum allowed by our institution). After which, all the dogs were adopted outside of the university and, from last report in June, 1998, are thriving as household pets.

It is recognized that there are a number of reasons for seeking a model that uses farm animals. Efforts are continuing to accomplish this. However, if the pig model cannot be adequately developed, then it is highly recommended that the canine model be used to complete this study.

CONCLUSIONS

IL-1 mediates inflammation in the CNS. It leads to a predominantly lymphocytic leukocytosis, similar to that observed in viral and autoimmune disease states. This effect occurs without systemic inflammation or fever.

IL-1 is more slowly eliminated from the CNS than previously suspected. It's mechanism of elimination is probably due to CSF resorption rather than proteolysis. Although slow, elimination alone does not account for the persistent CNS inflammatory state seen after IL-1 administration.

TNF also mediates inflammation in the CNS. After endogenous production and release, it contributes to the persistently lymphocytic leukocytosis characteristic of the IL-1 neuroinflammatory state.

IL-6 does not appear to contribute to the IL-1 mediated neuroinflammatory state.

This is the first direct evidence of cytokine mediation of the neuroinflammatory state. Our work demonstrates that the brain is not immunologically privileged and in fact responds in a specific manner to stimulation. Furthermore, under appropriate relevant physiologic conditions, the brain is immunocompetent as it can produce cytokines. It does this without systemic involvement.

As our work continues to evolve, it is our hope that this will translate into rational therapeutic approaches towards ameliorating the combat casualty condition of our fighting forces.

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Appendix I

Validation of the Cell-Dyn 3500 automated hematology analyzer

Canine CSF was obtained from i.c.v. shunts described previously (n=6). Sampling was at various times and under a variety of conditions such as shunt infection and post-operation. Immediately after sampling, a 130μl aliquot was assayed on the automated cell counter. From the same specimen, aliquots were processed using established procedures for manual determinations of RBC (n=105), WBC (n=90) and WBC differentials (n=28) counts. The investigator performing manual analysis was blinded to the results obtained by the automated cell counter.

Results demonstrate that WBC counts obtained by the automated cell counter were not significantly different from WBC counts obtained by manual counting using light microscopy with a hemacytometer. ($p>0.05$, paired t-test). Differential counts were also not significantly different for neutrophils, lymphocytes, monocytes or eosinophils ($p>0.05$, analysis of covariance linear regression). No basophils were identified by manual counting whereas the automated cell counter occasionally detected the presence of a low number of this WBC subspecies (0.8%$\pm$ 0.3 SEM). This discrepancy between manual and automated basophil counting is most likely a result of very low numbers in a relatively small sample volume. This is further complicated by any dilution used for manual processing since basophil granules are water soluble. As a consequence, the likelihood of observing any of these few cells is markedly reduced. In contrast, because the automated cell counter counts all the WBC in a given 130μl aliquot without further dilution, the likelihood of detecting any basophils is optimized. This method has the added advantage of demonstrating a population of basophils not currently recognized in cerebrospinal fluid. When compared to manual counting, RBC counts are significantly different by automated cell counter measurement ($p<0.05$, paired t-test). This discrepancy is most likely due to the different method employed by the automated cell counter in counting these cells, which is by electrical flow impedance rather than using the laser optical system employed for WBC.

In conclusion, the Cell-Dyn 3500 automated hematology cell counter is a rapid and reliable instrument for determining CSF total WBC and differential WBC (neutrophils, lymphocytes, monocytes and eosinophil) counts but not RBC counts.